

# Evidence that the mitochondrial activator of phosphorylated branched-chain 2-oxoacid dehydrogenase complex is the dissociated E<sub>1</sub> component of the complex

Stephen J. Yeaman, Kenneth G. Cook, Richard W. Boyd and Rowena Lawson

*Department of Biochemistry, The University, Newcastle upon Tyne NE1 7RU, England*

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Branched-chain 2-oxoacid dehydrogenase complex is inactivated by phosphorylation of the  $\alpha$ -subunit of the E<sub>1</sub> component of the complex. High-speed supernatant from rat liver mitochondria contains an activator protein which can restore activity to the phosphorylated complex without concomitant dephosphorylation [(1982) FEBS Lett. 147, 35–39]. We report here several lines of evidence which indicate that activator is the dissociated non-phosphorylated form of the E<sub>1</sub> component.

<i>Branched-chain 2-oxoacid dehydrogenase complex</i>	<i>Phosphorylation activator</i>	<i>E<sub>1</sub> component</i>
<i>(Rat liver mitochondria)</i>		

## 1. INTRODUCTION

The mitochondrial branched-chain 2-oxoacid dehydrogenase complex catalyses a rate-limiting step in the oxidation of the essential branched-chain amino acids [1]. The complex comprises a central core of a transacylase component (E<sub>2</sub>) around which are arranged the 2-oxoacid dehydrogenase (E<sub>1</sub>) and dihydrolipoyl dehydrogenase (E<sub>3</sub>) components. E<sub>1</sub> consists of two non-identical subunits termed  $\alpha$  and  $\beta$  [2].

The activity of the complex is regulated by reversible phosphorylation. Inactivation results from phosphorylation of the  $\alpha$ -subunit of the E<sub>1</sub> component by a protein kinase which is intrinsic to the complex [3,4]. As with the pyruvate dehydrogenase complex, which is regulated in an analogous manner [5], the phosphorylated form of the branched-chain 2-oxoacid dehydrogenase complex is essentially inactive under all assay conditions and no allosteric activators of the phosphorylated form are known.

Recently however a protein 'activator' of phosphorylated branched-chain 2-oxoacid dehydrogenase complex has been reported in high-speed

supernatant from rat liver and kidney mitochondria [6]. The factor, which is apparently absent from heart and muscle mitochondria, can restore full activity to the phosphorylated complex without dephosphorylation, re-activation being essentially instantaneous. Levels of the activator are sufficiently high as to lead to the suggestion that the activator may be of physiological significance and its absence from heart (and skeletal muscle) has been postulated as a partial explanation of the different regulation of the complex in liver and kidney as opposed to muscle [1,6].

Here, we report several pieces of evidence that the activator protein is in fact free E<sub>1</sub> component which has presumably dissociated from the complex during ultracentrifugation. A physiological role for the activator must therefore be seriously questioned.

## 2. MATERIALS AND METHODS

Branched-chain 2-oxoacid dehydrogenase complex from bovine kidney cortex was purified and assayed as in [7]. The specific activity of the final preparation was approx. 5 units/mg. Phosphor-

ylated complex was prepared as in [7]. Unless otherwise indicated no attempt was made to remove the ATP-Mg from the final preparation. E<sub>2</sub>-kinase sub-complex was obtained by resolution of the complex in high concentrations of sodium chloride (unpublished and [2]). The E<sub>2</sub>-kinase sub-complex was devoid of overall catalytic activity (even in the presence of added E<sub>3</sub> component) and the  $\alpha$ - and  $\beta$ -subunits of the E<sub>1</sub> component were completely absent, as indicated by polyacrylamide gel electrophoresis in the presence of SDS.

Mitochondria from rat liver, kidney and heart were prepared essentially as in [8], using 0.25 M sucrose, 10 mM Tris-HCl (pH 7.3) as homogenisation buffer. They were then washed in 20 mM sodium phosphate, 0.1 mM EDTA, 15 mM  $\beta$ -mercaptoethanol (pH 7.3) and frozen, using liquid N<sub>2</sub>, at a protein concentration of approx. 25 mg/ml. After freeze-thawing twice, high-speed supernatant was obtained by centrifugation for 2 h at  $180000 \times g_{av}$  at 4°C. This supernatant was devoid of pyruvate dehydrogenase and branched-chain 2-oxoacid dehydrogenase activities.

Activator in high-speed supernatant was assayed by addition to a reaction cuvette containing all the constituents necessary for assay of branched-chain 2-oxoacid dehydrogenase plus either phosphorylated inactive branched-chain 2-oxoacid dehydrogenase complex (50  $\mu$ g) or E<sub>2</sub>-kinase sub-complex (50  $\mu$ g).

### 3. RESULTS

In agreement with [6], we have observed that high-speed supernatant from rat liver and kidney mitochondria (but not heart) contains an activator protein which can restore full activity to phosphorylated branched-chain 2-oxoacid dehydrogenase complex (not shown). All subsequent data refer to work with rat liver mitochondria but similar observations have been made using rat kidney. The activator can be precipitated by (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (45% saturation) but is relatively labile, its activity being destroyed by incubation at 30°C for 1 h or by storage overnight in dilute solution at 4°C. However, after precipitation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> it can be stored in (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> at -20°C for several days without appreciable loss of activity.

One possibility which seemed to merit investigation was that activator protein is in fact dissociated E<sub>1</sub> component, which can recombine with the E<sub>2</sub> component of phosphorylated inactive complex to generate the overall catalytic activity of the complex. This was tested using E<sub>2</sub>-kinase sub-complex which was devoid of E<sub>1</sub>. As can be seen in fig.1, addition of a preparation of activator to excess E<sub>2</sub> (in the presence of excess E<sub>3</sub>) generates significant catalytic activity. This occurs essentially instantaneously and the amount of activity achieved is proportional to the amount added of activator protein in the presence of excess E<sub>2</sub> (fig.1) or to the amount of E<sub>2</sub> in the presence of excess activator protein (not shown).

If the activator protein is in fact the E<sub>1</sub> component it should exhibit properties similar to those of E<sub>1</sub>. Transition-state analogues of thiamine pyrophosphate are potent inhibitors of the E<sub>1</sub> components of the 2-oxoacid dehydrogenase complexes [9,10]. One such analogue, thiamine thiothiazolone pyrophosphate (TTTPP), strongly inhibits bovine kidney branched-chain 2-oxoacid dehydrogenase complex, 50% inhibition being

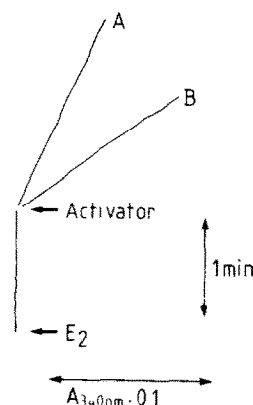


Fig.1. Effect of activator protein on the branched-chain 2-oxoacid dehydrogenase activity of the purified E<sub>2</sub> component of the complex. Taken from trace from a Cecil 272 linear readout spectrophotometer linked to a chart recorder. The cuvette contained (in 1 ml) the appropriate substrates and co-factors (including E<sub>3</sub>) for assay of branched-chain 2-oxoacid dehydrogenase [7]. E<sub>2</sub> (5  $\mu$ g) and activator, (A) 15  $\mu$ g protein; (B) 60  $\mu$ g protein, purified by precipitation using (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, were added as indicated. The activator preparation had no detectable branched-chain 2-oxoacid dehydrogenase activity (not shown).

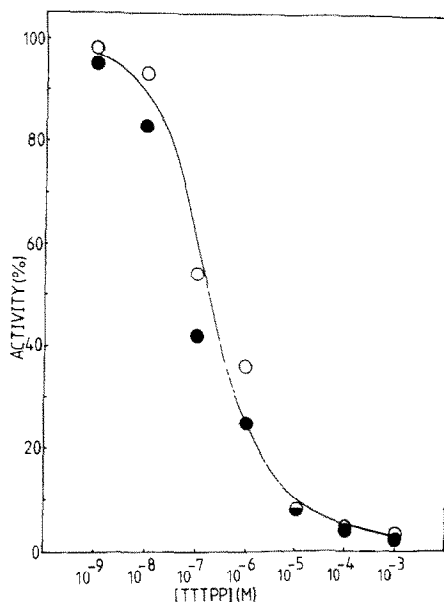


Fig. 2. Effect of thiamine thiothiazolone pyrophosphate (TTTPP) on activity of branched-chain 2-oxoacid dehydrogenase complex and of activator protein. Samples (purified complex or mitochondrial high-speed supernatant) were pre-incubated for 5 min at 20°C with the indicated concentration of TTTPP and then aliquots (10  $\mu$ l) were assayed for branched-chain 2-oxoacid dehydrogenase activity. Activator in the high-speed supernatant was assayed in the presence of excess  $E_2$  (50  $\mu$ g) and  $E_3$ . Branched-chain 2-oxoacid dehydrogenase complex (●), activator protein (○). Addition of  $10^{-5}$  M TTTPP directly to a reaction cuvette had no significant effect on the activity of a sample of complex.

observed at approx.  $10^{-7}$  M (fig. 2). The activator protein is also inhibited by TTTPP, inhibition being observed over the same range of concentrations of TTTPP (fig. 2).

A key property of  $E_1$  is that, at least when bound to the complex, it can be phosphorylated and inactivated by a specific protein kinase intrinsic to the complex [3,4,11]. We therefore investigated the effect of the kinase on the properties of the activator, using phosphorylated branched-chain 2-oxoacid dehydrogenase complex as a source of kinase. When phosphorylated complex is incubated with high-speed supernatant in the presence of ATP-Mg, the activity of the activator is lost. The loss of activator is a time-dependent, ATP-dependent process, indicative of the involve-

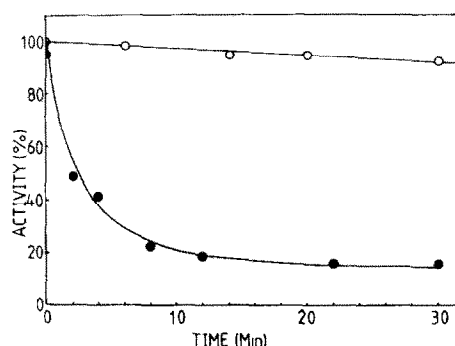


Fig. 3. Inactivation of activator protein under conditions favouring phosphorylation. Mitochondrial high-speed supernatant (4.5 mg protein, containing 0.08 units of activity when assayed in the presence of phosphorylated complex) was incubated at 20°C with excess phosphorylated (inactive) branched-chain 2-oxoacid dehydrogenase complex (600  $\mu$ g, added as a source of kinase and to allow quantitation of activator) in a final volume of 300  $\mu$ l sodium phosphate (30 mM, pH 7.3), containing EDTA (0.1 mM),  $\beta$ -mercaptoethanol (15 mM), NaF (50 mM), oligomycin B (10  $\mu$ g/ml),  $MgCl_2$  (10 mM) and ATP (0.5 mM). Aliquots (40  $\mu$ l) were removed at the indicated times and assayed for branched-chain 2-oxoacid dehydrogenase activity (●). In the control incubation (○), ATP was omitted and the phosphorylated complex was pre-treated with glucose and hexokinase [7] to scavenge any residual ATP.

ment of a phosphorylation mechanism (fig. 3). When  $E_2$ -kinase complex (devoid of  $E_1$ ) is incubated with high-speed supernatant in the presence of [ $\gamma$ - $^{32}P$ ]ATP-Mg, a polypeptide of  $M_r \approx 46000$  becomes phosphorylated (fig. 4). This subunit size corresponds to that of the  $\alpha$ -subunit of the  $E_1$  component [2].

#### 4. DISCUSSION

Here we confirm the basic observations in [6] that high-speed supernatant from rat liver (and kidney but not heart) mitochondria contains a protein activator which can activate the phosphorylated form of the branched-chain 2-oxoacid dehydrogenase complex. However, we present several pieces of evidence which indicate that the activator is in fact free dissociated  $E_1$  component of the complex. The strongest evidence in support of this is the observation that activator from high-

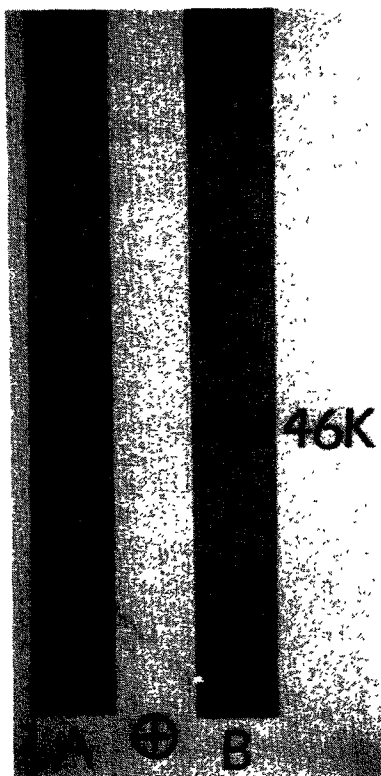


Fig.4. Phosphorylation of mitochondrial high-speed supernatant by  $E_2$ -kinase sub-complex. High-speed supernatant ( $450 \mu\text{g}$  protein) was incubated at  $20^\circ\text{C}$  for 30 min with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  ( $0.5 \text{ mM}$ ,  $200 \text{ cpm/pmol}$ ),  $\text{MgCl}_2$  ( $10 \text{ mM}$ ),  $\text{NaF}$  ( $50 \text{ mM}$ ), oligomycin B ( $10 \mu\text{g/ml}$ ) in a total volume of  $30 \mu\text{l}$  in the absence (A) and presence (B) of  $E_2$ -kinase ( $30 \mu\text{g}$ ). An aliquot ( $10 \mu\text{l}$ ) was then analysed by SDS-polyacrylamide gel electrophoresis and autoradiography as in [7]. No phosphorylation was detected using  $E_2$ -kinase in the absence of high-speed supernatant (not shown).

speed supernatant can confer on  $E_2$ - $E_3$  sub-complex the ability to oxidise branched-chain 2-oxoacids (fig.1). This demonstrates that high-speed supernatant contains active  $E_1$  component which can interact with  $E_2$  and generate overall catalytic activity (in the presence of  $E_3$ ). It seems likely therefore that  $E_1$  can interact with the  $E_2$  core of phosphorylated inactive complex to restore overall catalytic activity. We have recently been able to demonstrate that homogeneous preparations of  $E_1$ , obtained by resolution of the purified complex, can restore activity to the phosphorylated complex or  $E_2$  component (unpublished). It is

not yet clear whether  $E_1$  acts by displacing phosphorylated  $E_1$  from the complex or by binding to unoccupied sites on the  $E_2$  core. Detailed studies using purified complex and components will be necessary to resolve this question. Further evidence that activator protein is  $E_1$  includes the observation that activator 'activity' is lost following pre-treatment with a transition state analogue of thiamine pyrophosphate, an essential co-factor for  $E_1$  (fig.2). Furthermore, activator 'activity' is destroyed in a time-dependent manner by incubation with ATP-Mg and a source of the kinase considered to be specific for  $E_1$  of the branched-chain 2-oxoacid dehydrogenase complex (fig.3). Loss of activity is apparently associated with phosphorylation of a polypeptide of  $M_r \approx 46000$ , although we are not yet able to exclude totally the possibility that other minor polypeptides in the high-speed supernatant become phosphorylated and are associated with loss of activator function. However, this seems unlikely.

Failure to detect the activator in supernatant from heart mitochondria may be explained by the observation that, in heart, the complex (and therefore free  $E_1$ ) is predominantly in the phosphorylated, inactive form.

We do not yet know whether  $E_1$  found in mitochondrial high-speed supernatant has fallen from the complex during preparation of the extract and subsequent ultracentrifugation, as is the case with the  $E_3$  component [2], or else whether free dissociated  $E_1$  exists within the mitochondria. In either case it is difficult to envisage a regulatory role for this free  $E_1$  activity.

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